

Human circulating influenza-CD4⁺ ICOS1⁺IL-21⁺ T cells expand after vaccination, exert helper function, and predict antibody responses

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Protection against influenza is mediated by neutralizing antibodies, and their induction at high and sustained titers is key for successful vaccination. Optimal B cells activation requires delivery of help from CD4⁺ T lymphocytes. In lymph nodes and tonsils, T-follicular helper cells have been identified as the T cells subset specialized in helping B lymphocytes, with interleukin-21 (IL-21) and inducible costimulatory molecule (ICOS1) playing a central role for this function. We followed the expansion of antigen-specific IL-21⁺ CD4⁺ T cells upon influenza vaccination in adults. We show that, after an overnight in vitro stimulation, influenza-specific IL-21⁺ CD4⁺ T cells can be measured in human blood, accumulate in the CXCR5⁻ICOS1⁺ population, and increase in frequency after vaccination. The expansion of influenza-specific ICOS1⁺IL-21⁺ CD4⁺ T cells associates with and predicts the rise of functionally active antibodies to avian H5N1. We also show that blood-derived CXCR5⁻ICOS1⁺ CD4⁺ T cells exert helper function in vitro and support the differentiation of influenza specific B cells in an ICOS1- and IL-21-dependent manner. We propose that the expansion of antigen-specific ICOS1⁺IL-21⁺ CD4⁺ T cells in blood is an early marker of vaccine immunogenicity and an important immune parameter for the evaluation of novel vaccination strategies.

CD4 help | predictivity | humoral response

To confer protection, human vaccines rely on the induction of neutralizing antibodies and on the generation of a pool of memory lymphocytes able to mount an accelerated response upon encounter with the target pathogen. In recent years, novel vaccines, adjuvants, and delivery systems that are able to improve vaccine immunogenicity while reducing their reactogenicity have been developed. As vaccines are given to healthy subjects, their development is a challenging endeavor that requires extensive studies to assess safety, immunogenicity, and clinical efficacy. To accelerate the screening of novel candidates, research has focused on the identification of early biomarkers, molecular and transcriptional signatures predicting vaccine efficacy (1). Predictors should be easy to test in large clinical trials and have a clear mechanistic relationship with the correlates or surrogates of protection taken as the study endpoint. We have previously shown that an early postvaccination increase in the number of vaccine-specific CD4⁺ T cells is correlated in a predictive manner with the rise and long-term maintenance of protective antibody titers to avian influenza (2). The aim of the present study was to characterize the CD4⁺ T cells subset responsible for this function.

T follicular helper (T_{fh}) cells have been identified in lymph nodes and tonsils as the CD4⁺ T cells subpopulation specialized in providing help to B cells (3–11). The recent identification of a circulating counterpart of this T cells subset in blood led us to investigate whether vaccine-specific IL-21⁺ CD4⁺ T cells are detectable in human blood, if their frequency is modulated by vaccination, and if their expansion correlates with an increase in

functional antibodies (12). We analyzed samples from a clinical study in which healthy adults were immunized with one dose of MF59-adjuvanted avian H5N1 followed by a dose of adjuvanted tetravalent influenza vaccine containing H5N1 and three seasonal influenza strains. We show that after overnight in vitro stimulation, required to detect antigen specific T cells and dissect their cytokine profile, influenza specific IL-21 positive (IL-21⁺) CD4⁺ T cells are detectable in adult blood, expand in number after vaccination and accumulate in the CXCR5⁻ICOS1⁺ subset. We find that the expansion of vaccine specific ICOS1⁺IL-21⁺ CD4⁺ T cells predicts the postvaccination rise of functional antibodies in these individuals. Finally, we show that blood-derived CXCR5⁻ICOS1⁺ CD4⁺ T cells are enriched in T cells able to help influenza-specific B cells differentiation into antibody-secreting cells in vitro in an IL-21- and ICOS1-dependent manner. We suggest that the expansion of the antigen-specific ICOS1⁺IL-21⁺ CD4⁺ T cells subset in blood is an early predictor of a vaccine's ability to stimulate humoral immunity and a useful surrogate measure of vaccine's immunogenicity in humans.

Results

Two Vaccinations Are Required to Increase Hemagglutination Inhibition Antibody Titers to H5N1 Whereas One Is Sufficient for H3N2. Healthy adults were immunized with two doses of MF59-adjuvanted H5N1 and one dose of 2007 to 2008 seasonal influenza vaccines. The antibody response to vaccination was tested by hemagglutination inhibition (HI) at baseline and 3 wk after each vaccination. In agreement with previous studies (2, 13), two doses of MF59-adjuvanted H5N1 vaccine were required to induce a significant level of HI antibodies to the H5N1 avian influenza strain, whereas only one vaccination was sufficient for a substantial increase of HI titers to seasonal H3N2 (Fig. 1). The absence of baseline titers, as well as the requirement for two vaccinations, confirms the lack of exposure of this study population to H5N1 in comparison with the serological evidence of preexposure to H3N2.

One Vaccination Is Sufficient to Induce the Expansion of H5N1- and H3N2-Specific CD4⁺ T Cells. We then analyzed the frequency of cytokine-producing CD4⁺ T cells (any combination of IL-2, TNF- α ,

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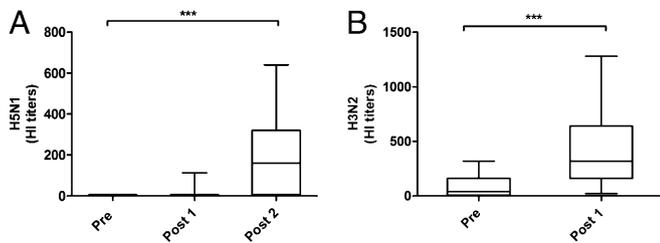


Fig. 1. HI antibody response to vaccination. HI antibody responses to the (A) H5N1 and (B) H3N2 vaccine strains at baseline (Pre), 21 d after the first dose (Post 1), and 21 d after the second dose (Post 2) of respective vaccine. The lines crossing the box plots represent median titers, and whiskers indicate the minimum and maximum values (***P* < 0.0001 by Tukey–Kramer test).

IFN- γ , and IL-21) after overnight in vitro stimulation with subunit H5N1 or H3N2, hereafter referred to as total influenza-specific CD4⁺ T cells. Unlike the antibody response (HI), one vaccination was sufficient to expand H5N1- and H3N2-specific CD4⁺ T cells (after one vaccination [“post 1”] vs. before vaccination [“pre”], *P* < 0.0001). The frequency of H5N1-specific CD4⁺ T cells increased only moderately after the second vaccine dose (Fig. 2A). After one vaccination, CD4⁺ T cells specific for H5N1 and H3N2 had a similar cytokine profile, and the response was dominated by single (white) and double (striped) cytokine-producing T cells (synthesizing IL-2 and/or TNF- α ; Fig. 2B) (14).

Influenza Vaccination Induces the Expansion of H5N1- and H3N2-Specific IL-21⁺ CD4⁺ T Cells. We then asked whether influenza-specific CD4⁺ T cells able to synthesize IL-21 cytokine (IL-21⁺ CD4⁺ T cells) were detectable in blood after overnight stimulation with vaccine antigens, and whether their number was increased after vaccination. We found a significant expansion of H5N1-specific IL-21⁺ CD4⁺ T cells after one dose of vaccine (post 1 vs. pre, *P* = 0.005) and a further increase after the second vaccination (“post 2” vs. post 1, *P* = 0.02). Similarly, one vaccination was sufficient to induce a significant expansion of H3N2-specific IL-21⁺ CD4⁺ T cells (*P* = 0.0001; Fig. 3A and Fig. S1). After one vaccination, the majority of antigen-activated CD4⁺ T cells, specific for H5N1 or H3N2, secreted IL-21⁺ with additional cytokines, and this profile did not change after the second dose of the H5N1 vaccine (Fig. 3B). The difference in the cytokine profile between total influenza-specific CD4⁺ T cells (primarily composed of T cells secreting one or two cytokines; Fig. 2B) and influenza-specific IL-21⁺ CD4⁺ T cells (primarily comprised of cells secreting three or four cytokines; Fig. 3B) suggests that IL-21⁺ T cells represent a specialized subset enriched in multifunctional T cells.

Circulating Influenza-Specific IL-21⁺ CD4⁺ T Cells Are CXCR5⁺ICOS1⁺ and Enriched in PD1⁺. We then investigated the expression of CXCR5 and ICOS1 on influenza-specific CD4⁺ T cells in peripheral blood mononuclear cells (PBMCs) from the clinical study. We found that, upon overnight in vitro stimulation with vaccine antigens, most of the H5N1- and H3N2-specific IL-21⁺ CD4⁺ T cells expressed ICOS1 but were negative for CXCR5. The second most abundant influenza-specific IL-21⁺ CD4⁺ T cells population was negative for CXCR5 and ICOS1, and only a minority of influenza-specific IL-21⁺ CD4⁺ T cells expressed CXCR5 (Fig. 4). The phenotype and cytokine profile of H5N1-specific IL-21⁺ CD4⁺ T cells did not change after the second vaccination (Figs. S2 and S3).

We analyzed the expression of CD40L, PD1, Bcl-6, and CXCR3 on circulating influenza-specific CD4 T cells (15–17). After overnight stimulation, CD40L was expressed by all the influenza-specific CD4⁺ T cells; PD1⁺ cells were more abundant in the

IL-21⁺ CD4 T cell subset ($\geq 40\%$ of IL-21⁺ compared with $\leq 10\%$ of IL-21⁻) and CXCR3 was expressed by 30% of influenza-specific CD4⁺ T cells (either IL-21⁺ or IL-21⁻). Bcl-6 was not detectable in peripheral blood CD4⁺ T cells (17).

To verify if the expression of CXCR5 and ICOS1 was modulated by overnight in vitro restimulation, PBMCs from buffy coats were sorted by the expression of CXCR5 and ICOS1 before stimulation with influenza antigen. CXCR5⁻ICOS1⁻ cells were the most abundant ($\leq 85\%$), followed by CXCR5⁻ICOS1⁺ ($\geq 10\%$) and CXCR5⁺ICOS1⁺ ($\leq 0.5\%$; Fig. S4). When the same number of sorted CD4⁺ T cells were stimulated in vitro with subunit influenza (i.e., H3N2) and autologous antigen-presenting cells (i.e., monocytes), the relative frequency of influenza-specific CD4⁺ T cells was equivalent in the three sorted populations (2.2% of CXCR5⁻ICOS1⁻, 2.7% CXCR5⁻ICOS1⁺, and 1.8% of CXCR5⁺ICOS1⁺). Taking into account the differences in the relative abundance of the three subsets in the total CD4⁺ T cells population, most influenza-specific CD4⁺ T cells were CXCR5⁻ICOS1⁻ (88%), followed by CXCR5⁻ICOS1⁺ (11%) and CXCR5⁺ICOS1⁺ ($\leq 1\%$). Secretion of IL-21 was instead restricted to CXCR5⁻ICOS1⁻ (55%) and CXCR5⁻ICOS1⁺ (45%) influenza-specific CD4⁺ T cells. We conclude that in vitro stimulation does not grossly modify the distribution of influenza-specific CD4⁺ T cells.

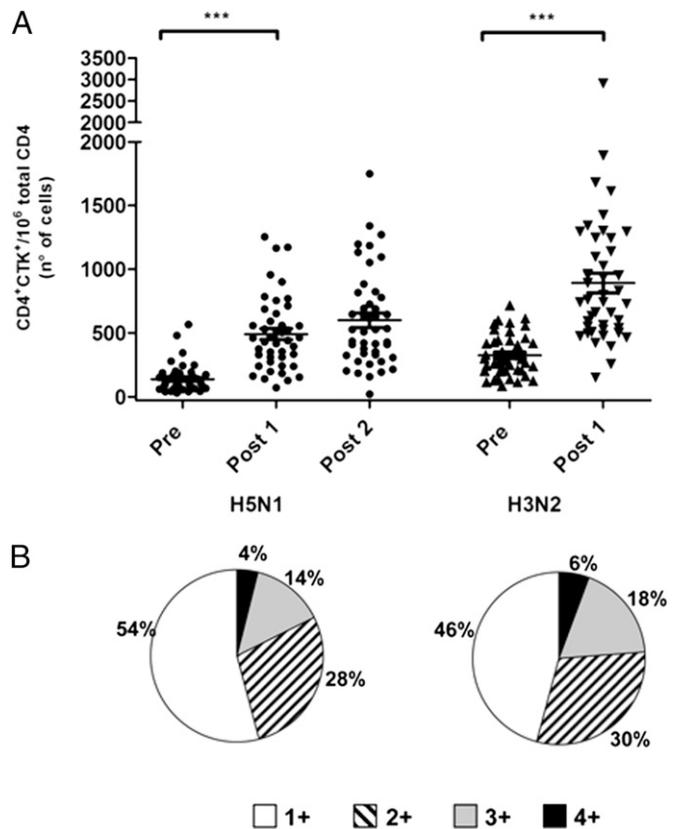


Fig. 2. Expansion and cytokine profile of H5N1- and H3N2-specific CD4⁺ T cells after vaccination. Total cytokine positive (CTK⁺) CD4⁺ T cells in PBMCs of vaccines after overnight in vitro stimulation with H5N1 (Left) or H3N2 (Right). (A) Number of CTK⁺ CD4⁺ T cells in PBMCs collected before (Pre), 3 wk after the first vaccination (Post 1), and 3 wk after the second vaccination (Post 2). Data are expressed as number of CTK⁺ CD4⁺/10⁶ CD4⁺ T cells \pm SEM (*n* = 44). (B) Cytokine profile of influenza-specific total CD4⁺ T cells 3 wk after the first vaccination (Post 1). The pie charts show the relative proportions of CD4⁺ T cells producing one (white), two (striped), three (gray), or four (black) cytokines. Shown is the average distribution in the whole database (*n* = 44; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, Tukey–Kramer test).

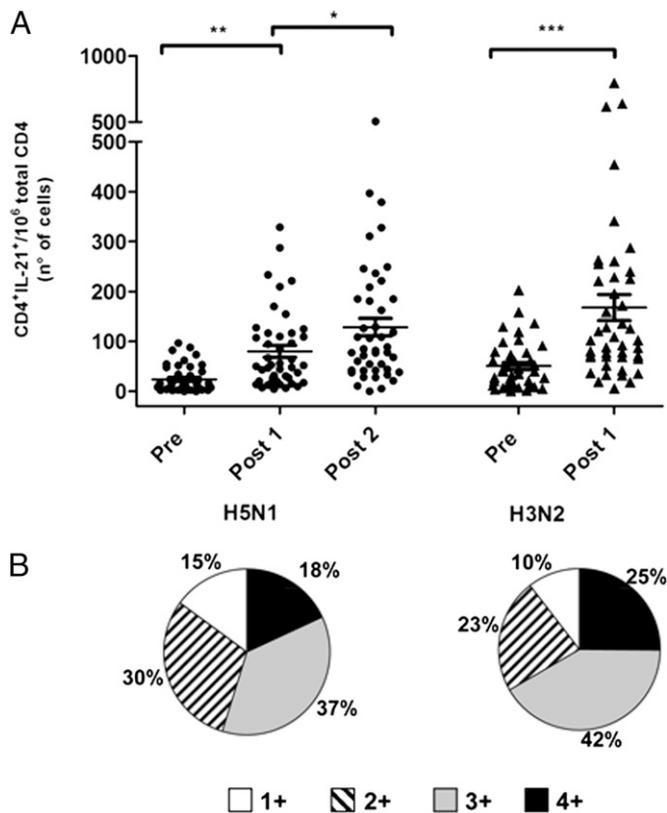


Fig. 3. Expansion and cytokine profile of IL-21⁺ H5N1- and H3N2-specific CD4⁺ T cells after vaccination. IL-21⁺ CD4⁺ T cells in PBMCs of vaccinees after overnight *in vitro* stimulation with H5N1 (Left) or H3N2 (Right). (A) Numbers of IL-21⁺ CD4⁺ after overnight stimulation with H5N1 or H3N2 before (Pre), 3 wk after the first vaccination (Post 1), and 3 wk after the second vaccination (Post 2). Data are expressed as number of antigen-specific IL-21⁺ CD4⁺/10⁶ CD4⁺ T cells \pm SEM ($n = 44$). (B) The pie charts show the relative Post 1 proportions of IL-21⁺ CD4⁺ T cells synthesizing IL-21 alone (white) or in combination with one (striped), two (gray), or three (black) additional cytokines. Shown is the average distribution in the whole database ($n = 44$; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, Tukey–Kramer test).

Expansion of Peripheral H5N1 Specific ICOS1⁺IL-21⁺ CD4⁺ T Cells Correlates with Postvaccination Increase in HI Antibody Titers. We tested whether the expansion after vaccination of influenza specific ICOS1⁺IL-21⁺, ICOS1⁻IL-21⁺, and total influenza-specific CD4⁺ T cells associated with the increase in functional antibodies. We adopted as a target HI antibody titers $\geq 1:80$, as it is the accepted correlate of protection in influenza (18), and as predictor a threefold expansion of vaccine-specific CD4⁺ T cells, based on the results from a rank correlation analysis of antibody and T cells responses to vaccination done in our previous study (13). After the first vaccination, at least a threefold expansion of H5N1 ICOS1⁺IL-21⁺ CD4⁺ T cells was the only parameter that predicted the increase of specific serum HI antibodies to titers $\geq 1:80$ after the second dose of vaccine (accuracy of 75%; $P = 0.02$, two tailed Fisher test; Fig. 5A). The association between the expansion of H5N1 ICOS1⁺IL-21⁺ CD4⁺ T cells and HI titers became even more robust after the second vaccination (accuracy of 82%; $P = 0.0006$; Fig. 5D). No significant association was found between the expansion of ICOS1⁻IL-21⁺ CD4⁺ T cells, after one or two doses, and HI titers $\geq 1:80$ ($P = 0.75$ and $P = 0.09$, respectively; Fig. 5B–E). Finally, the expansion of total H5N1 CD4⁺ T cells following one vaccination did not associate with HI titers $\geq 1:80$ ($P = 0.1$); however, the association become significant after the second dose (accuracy of 80%; $P = 0.001$; Fig. 5C–F). Rank correlation analysis confirmed the association

between H5N1-specific ICOS1⁺IL-21⁺ CD4⁺ T cells or total H5N1⁺ CD4⁺ T cells after the second vaccination (either as absolute number or fold increase vs. baseline) and HI titers (either as absolute titers or fold increase vs. baseline; Spearman $\rho = 0.5$, $P = 0.0006$ for ICOS1⁺IL-21⁺; $\rho = 0.5$, $P = 0.0003$ for total H5N1⁺ CD4⁺ T cells). No correlation was found between H5N1 ICOS1⁻IL-21⁺ CD4⁺ T cells and HI titers (Spearman $\rho = 0.3$, $P = 0.06$).

We then investigated the rank correlation between expansion of H3N2 CD4⁺ T cells and HI titers for seasonal H3N2. We found a weak correlation between fold change in HI titer and absolute numbers of ICOS1⁺IL-21⁺ CD4⁺ T cells (Spearman $\rho = 0.4$, $P = 0.009$). No correlation was found between HI titers and the expansion of H3N2 ICOS1⁻IL-21⁺ (Spearman $\rho = 0.2$, $P = 0.24$) or total H3N2 CD4⁺ T (Spearman $\rho = 0.3$, $P = 0.05$).

Peripheral Blood CXCR5⁻ICOS1⁺ CD4⁺ T Cells Provide Cognate Help to Antigen Specific B Cells *In Vitro*. Finally, we compared the ability of different blood-derived influenza-specific CD4⁺ T cells subsets to help B cells differentiate into antibody-secreting cells *in vitro*. Because of the limited amount of clinical samples, these experiments were performed with buffy coats. The frequency and phenotype of influenza-specific CD4⁺ T cells in the individual buffy coats was measured after overnight stimulation (Table S1). For the helper assay, to mimic the conditions used to identify antigen-specific CD4⁺ T cells in the clinical study, PBMCs were stimulated overnight with H3N2 before sorting on the expression of CXCR5 and ICOS1 (CXCR5⁻ICOS1⁺, CXCR5⁺ICOS1⁺, and CXCR5⁻ICOS1⁻ subsets). Sorted CD4⁺ T cells were rested for 10 d before coculture with autologous B cells. The Ig content was measured in the supernatant after 10 d of T-B cells coculture in the absence or presence of subunit H3N2. In three independent experiments, only CXCR5⁻ICOS1⁺ CD4⁺ T cells were able to support autologous B cells differentiation into antibody-secreting cells, measured by secretion of IgG and IgM (Fig. 6A–F). Antibodies were not detectable if B cells were cultured with the antigen but in the absence of CD4⁺ T cells (Fig. 6A–F, right bars of graphs). In addition, only the CXCR5⁻ICOS1⁺ CD4⁺ T cells supported the secretion of influenza-specific IgG and IgM by autologous B cells (Fig. 6G and H). The accumulation of influenza-specific IgG and IgM was reduced by more than 90% by blocking IL-21 or ICOS1, thus confirming the critical role of both pathways in the delivery of T cells help to B cells (Fig. 6G and H). Note that the concentration of H3N2-specific IgG and IgM was only a fraction of the total Ig detected in the cultures. It is reasonable to hypothesize that the addition of soluble antigens as a stimulus induced the formation of immune complexes that interfered with the detection of influenza-specific antibodies; thus, the assay may underestimate the amount of specific antibodies present in the cultures.

To exclude that CD4⁺ T cells helped B cells in a bystander, antigen-unrelated manner, we measured the content of antibodies specific for diphtheria toxoid (DT; CRM-197) in the T-B cells coculture supernatant. Upon stimulation with influenza antigens, we did not detect antibodies specific for CRM despite the presence in the buffy coat of DT-specific B cells that could be induced to secrete CRM specific antibodies upon polyclonal stimulation via CpG and IL-2 (Fig. 6I).

We conclude that, under the experimental conditions used, blood-derived influenza-specific CXCR5⁻ICOS1⁺IL-21⁺ CD4⁺ T cells help the *in vitro* differentiation of autologous B cells into cells secreting influenza-specific antibody in an antigen-, IL-21-, and ICOS1-dependent manner.

Discussion

The use of vaccines has proven to be a very successful medical intervention in the reduction of infectious diseases. Despite advancements in vaccine safety and efficacy, only a few novel

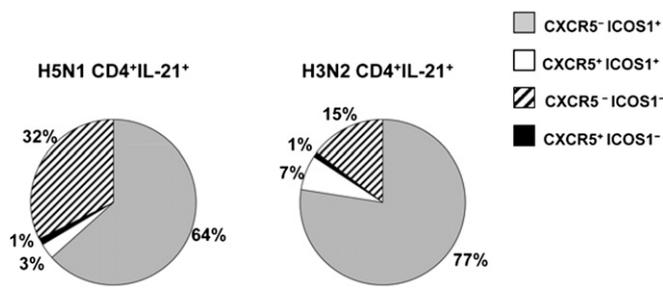


Fig. 4. Distribution of blood antigen-specific IL-21⁺ CD4⁺ T cells according to the expression of ICOS1 and CXCR5. Shown are the relative proportions of IL-21⁺ CD4⁺ T cells expressing CXCR5⁻ICOS1⁺ (gray), CXCR5⁺ICOS1⁺ (white), CXCR5⁻ICOS1⁻ (striped), and CXCR5⁺ICOS1⁻ (black) after overnight stimulation with H5N1 (Left) or H3N2 (Right). Shown is the average distribution in 10 subjects from the A/T group, analyzed 3 wk after the first vaccination.

products are approved for clinical use. Impediments in the development of novel vaccine candidates come, in part, from the limited predictive power of animal models and the need for extensive, prolonged, and costly testing for safety and immunogenicity in humans. Such limitations have spurred the search for biomarkers, molecular and genetic signatures able to predict vaccine safety and immunogenicity that could increase our understanding of the immune system and allow vaccine candidates to be more rapidly screened (1). We searched for an early immunological readout of vaccination associated with and predictive of the increase of HI titers $\geq 1:80$, used as the correlate of protection for influenza (18, 19). We have previously shown that vaccination of healthy adults with adjuvanted avian H5N1 influenza induces the rapid expansion of vaccine-specific CD4⁺ T cells whose frequency correlates in a predictive manner with the increase and long-term persistence of neutralizing antibodies (2). Herein, we report the identification of the T cells

subset responsible for this function. Tfh cells have been identified as the CD4⁺ T cells population specialized in providing help to B lymphocytes in lymph nodes and tonsils (3, 9, 20). Despite the absence of markers exclusive to this T cells subset (5, 6, 10, 11, 21, 22), analysis of their phenotype in lymph nodes and tonsils has pointed to a central role of IL-21, CXCR5, and ICOS1 (3, 9, 12, 23). The difficulty in obtaining human samples other than blood, and the recent identification of a migratory counterpart of this T cells population, led us to investigate if antigen-specific CD4⁺ T cells with functional properties of Tfh cells are measurable in peripheral blood, and if the expansion in their number is associated with effective vaccination (12, 17).

Data presented in the present study show that CD4⁺ T cells, able to secrete IL-21⁺ after short in vitro activation with influenza antigens, are detectable in adult blood and expand in number after vaccination. The majority of circulating influenza-specific IL-21⁺ CD4⁺ T cells are CXCR5⁻ICOS1⁻ and CXCR5⁻ICOS1⁺, express CD40L, and are enriched in PD1⁺ cells, known to play a critical role in the activation and selection of high affinity B cells (16, 24). We observed at least a threefold expansion, after the first vaccination, of H5N1-specific ICOS1⁺IL-21⁺ CD4⁺ T cells that associates in a predictive manner with the increase of HI antibodies to protective levels. The expansion of total H5N1-specific CD4⁺ T cells was associated with the increase of functional antibodies to potentially protective levels only after the second vaccination. The expansion of H5N1-specific ICOS1⁻IL-21⁺ CD4⁺ T cells, despite being the most abundant subset, does not associate with the increase in HI antibodies at any time point analyzed, highlighting the central role of IL-21 and ICOS1 in the delivery of help to B cells. A weak correlation was also observed between the expansion of H3N2-specific ICOS1⁺IL-21⁺ CD4⁺ T cells and the increase in antibody titers. Furthermore, circulating CXCR5⁻ICOS1⁺ cells are the only CD4⁺ T cells subset able to support the in vitro differentiation of autologous B cells into cells secreting influenza-specific IgM and IgG in an IL-21- and ICOS1-dependent manner (3, 9, 12).

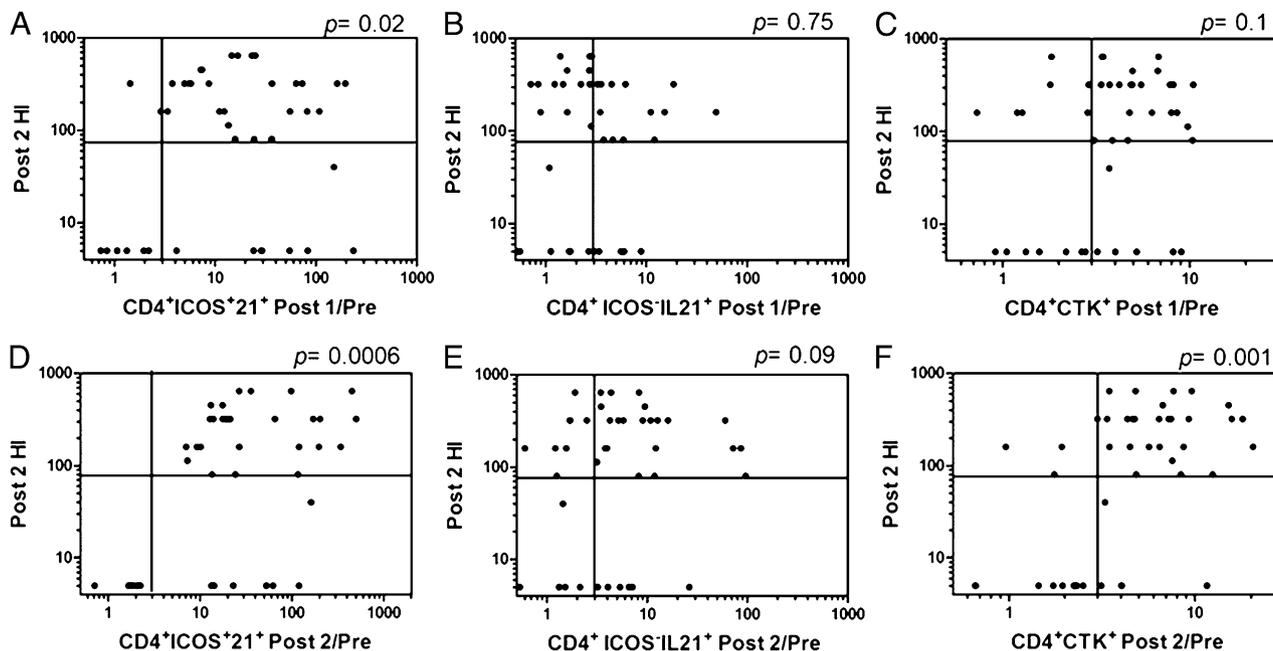


Fig. 5. Frequency of H5N1-specific ICOS1⁺IL-21⁺ CD4⁺ T cells at Post 1 significantly correlates with and predicts HI titers at Post 2. Associations between paired values of HI titers to H5N1 (Post 2) and fold increase of H5N1-specific CD4⁺ at (A–C) Post 1 vs. Pre or (D–F) at Post 2 vs. Pre. The association was calculated with fold increase in (A and D) H5N1 ICOS1⁺IL-21⁺ CD4⁺ T cells, (B and E) H5N1 ICOS1⁻IL-21⁺ CD4⁺ T cells, and (C and F) H5N1 total CTK⁺ CD4⁺ T cells. Fisher two-tailed exact test was used. Horizontal dashed lines indicate the value of HI titer of 80, the accepted threshold of protective antibodies. Vertical dashed lines indicate the value of threefold increase in H5N1 CD4⁺ T cells ($n = 44$).

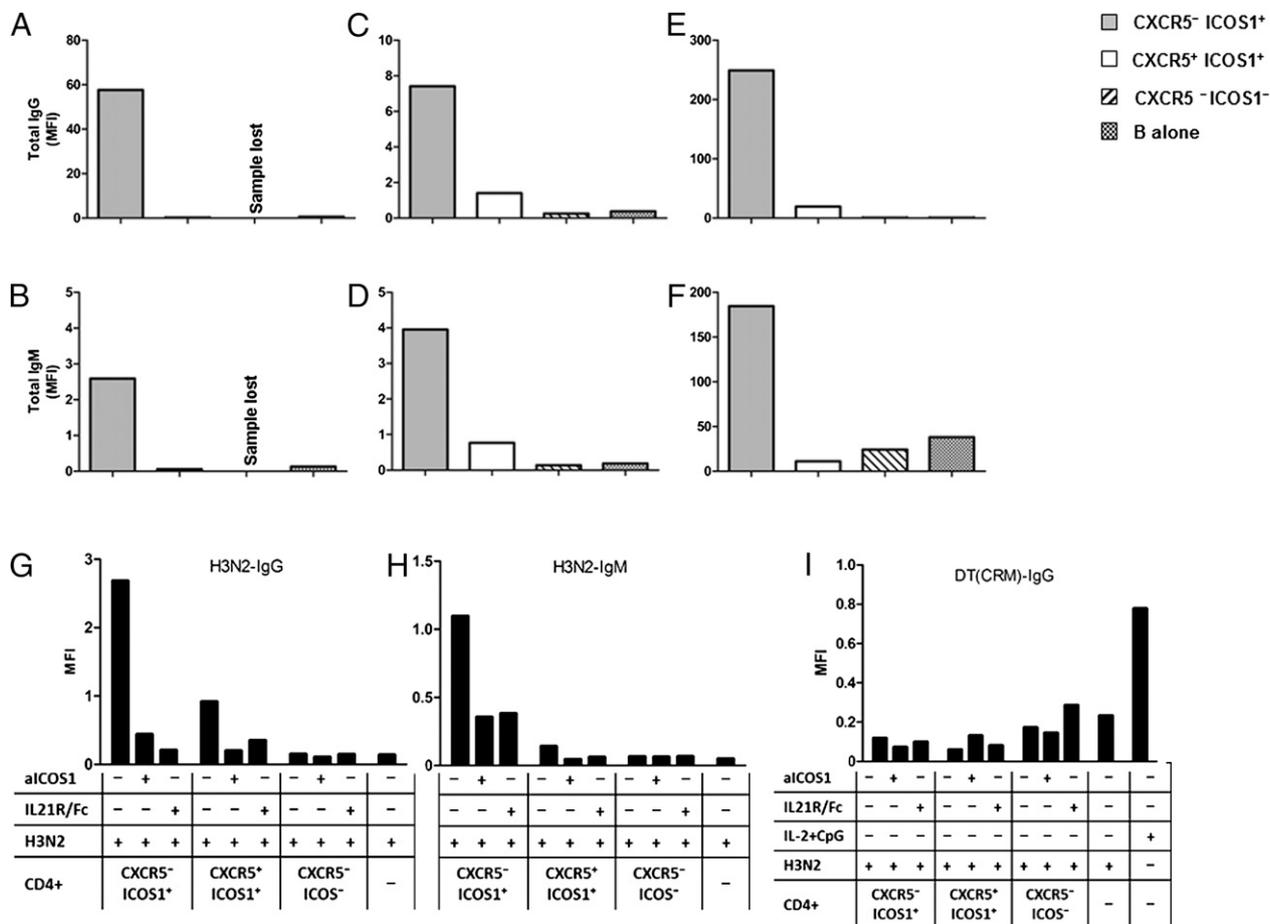


Fig. 6. Influenza-specific CXCR5⁻ ICOS1⁺ CD4⁺ T cells provide cognate help for antigen-specific antibody production in vitro. (A–F) Immunoglobulin content in day 10 supernatant from three independent experiments. Shown are total IgG (A, C, and E) and total IgM (B, D, and F). (G) H3N2-specific IgG, (H) H3N2-specific IgM, and (I) DT (CRM-197)-specific IgG content measured in the coculture supernatants of the experiment shown in E and F. The last column of I shows the content in DT (CRM)-specific IgG in the supernatants from B cells stimulated with CpG and IL-2 in the absence of CD4⁺ T cells. Values are reported as median fluorescence intensity (MFI).

While this paper was under revision, a paper describing similar findings was published (17). Both papers identify a population of blood CD4⁺ T cells with functional characteristics of Tfh cells whose expansion upon vaccination associates with an increase in antibody titers. Bentebibel et al. identified this population, 7 d after vaccination for seasonal influenza, as CXCR5⁺ ICOS1⁺ CXCR3⁺ CD4⁺ T cells. They show that the expansion in blood of this subset, independent of their antigen specificity, correlates with the fold increase of antibody titers in adults vaccinated with seasonal influenza. We addressed a similar question, but from a different perspective. We analyzed the frequency of influenza-specific IL-21⁺ CD4⁺ T cells and their distribution in different subsets relative to total CD4⁺ T cells, thus providing precise information on their prevalence in PBMCs. In addition, we monitored the kinetics of their expansion in response to avian H5N1, an antigen for which vaccinees are potentially naive, and to H3N2, for which the population has already been exposed. We show that the expansion of avian or seasonal influenza CXCR5⁻ ICOS1⁺ IL-21⁺ CD4⁺ T cells, 3 wk after vaccination, is associated with the increase in neutralizing antibodies. Our identification of CXCR5⁻ T cells as responsible for this function is in disagreement with previous results in mice and humans (3, 5, 9, 12, 17, 25). We believe the analysis of lymphoid tissue vs. blood, as well as differences in the experimental procedure, can explain such discrepancies. The in vitro stimulation step of frozen PBMCs vs. the analysis of whole blood ex vivo, the focus on antigen-specific

vs. total CD4⁺ T cells, as well as the analysis of different time points (day 21 vs. day 7) could account for such differences. Whether blood IL-21⁺ T cells are derived from the canonical Tfh cell population described in lymph nodes and tonsils is an intriguing question that neither paper is capable of elucidating given the impracticality of performing such complex experiments in humans. Additional studies that compare different antigens to which the study population is naive or has already been exposed, different vaccine formulations, and different age groups, as well as the analysis of the response at time points more distant from vaccination, will be required to more finely dissect the role of this T cells subset as an early predictor of vaccine immunogenicity.

Materials and Methods

Clinical Trial Design. Healthy adults (age ≥18 y) were randomized in three arms and received (i) one dose of subunit monovalent MF59-adjuvanted H5N1 (A/Vietnam/1194/2004 at 7.5 μg) followed, 3 wk later, by one dose of a tetravalent vaccine (MF59-H5N1 A/Vietnam/1194/2004 at 7.5 μg and H1N1 A/Solomon Islands/3/2006, H3N2 A/Wisconsin/67/2005, B/Malaysia/2506/2004 at 15 μg each; group A/T); (ii) one dose of a tetravalent vaccine followed, 3 wk later, by one dose of monovalent MF59-H5N1 (group T/A); or (iii) one dose of MF59-H5N1 in one arm and one dose of not-adjuvanted seasonal vaccine in the other arm, followed after 3 wk by one dose of monovalent MF59-H5N1 (group A-S/A; ClinicalTrials.gov identifier NCT00620815). All vaccines were prepared at Novartis Vaccines and Diagnostics. PBMCs for immunogenicity analysis were collected from 30 subjects randomly selected from each group. For the purpose of this study, 44 subjects from the T/A

($n = 24$), A-S/A ($n = 9$), and A/T ($n = 11$) groups were selected, based on the availability of PBMCs, and analyzed in detail for CD4⁺ T cells and antibody responses to vaccine antigens H5N1 and H3N2 at baseline and 3 wk following each vaccination.

HI Assay. Antibody titers to H5N1 and H3N2 were measured as previously described (26, 27).

Analysis of Influenza-Specific CD4⁺ T Cells. Frequency, phenotype, and cytokine profile of influenza-specific CD4⁺ T cells was analyzed by polychromatic flow cytometry after overnight in vitro stimulation of PBMCs with subunit influenza antigens and Brefeldin A as detailed elsewhere (2) and in *SI Materials and Methods*.

CD4⁺ T Cell–Antigen Presenting Cells (APC) in Vitro Coculture. Buffy coats were used for CD4⁺ T cell–APC in vitro coculture experiments. Autologous CD4⁺ T cell subpopulation (CXCR5⁺ICOS1⁺/CXCR5⁺ICOS1⁺/CXCR5⁺ICOS1[−]) and monocytes were sorted from unstimulated PBMCs and cocultured overnight in the presence of subunit H3N2 and Brefeldin A. Frequency of total influenza-specific and IL-21⁺ influenza-specific CD4⁺ T cells in the distinct T cells subsets was analyzed with polychromatic flow cytometry. Results are calculated taking into account the relative abundance of each CD4⁺ T cells subpopulation in the buffy coats used (*SI Materials and Methods*).

In Vitro Helper Assay. PBMCs from buffy coats were restimulated overnight with H3N2 before sorting CD4⁺ T cells based on the expression of ICOS1 and

CXCR5 into three populations (CD4⁺ CXCR5[−]ICOS1⁺, CD4⁺ CXCR5⁺ICOS1⁺, and CD4⁺ CXCR5[−]ICOS1[−]). Sorted T cells were rested for 10 d before coculture with the autologous B cells in the presence or absence of subunit H3N2, anti-ICOS1-L, and mAb blocking IL-21 (*SI Materials and Methods*).

Measurement of Total and Antigen-Specific Immunoglobulins in Culture Supernatants of the Helper Assay. Coculture supernatants were tested by ELISA by using Gyrolab technology (*SI Materials and Methods*).

Statistical Analysis. The Tukey–Kramer test was performed to analyze the mean HI titers or mean frequencies of CD4⁺ T cells after different vaccinations. The association between the fold increase in CD4⁺ T cells and HI titers was analyzed by Fisher two-tailed exact test. A threefold increase of H5N1 CD4⁺ T cells and HI antibody titers $\geq 1:80$ were chosen as thresholds based on the results from our previous studies (13). Spearman rank correlation analysis was performed between CD4⁺ T cells (either as absolute number and fold expansion vs. baseline) and HI titers (either as absolute titers and fold increase vs. baseline). Analysis was performed with JMP 8.0.1 software.

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